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Contract N00014-88-K-0401

Technical Report No. 008

Electron Transfer between Glucose Oxidase and Electrodes via Redox Mediators Bound with Flexible Chains to the Enzyme Surface

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Accepted for Publication in
Journal of the American Chemical Society
February 1991

January 11, 1991

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release and sale; its distribution is unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S) TECHNICAL REPORT NO. 008	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Dept. of Chemical Engineering University of Texas at Austin 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Department of Sponsored Projects University of Texas at Austin
6c. ADDRESS (City, State, and ZIP Code) Austin, TX 78712-1062	7b. ADDRESS (City, State, and ZIP Code) P.O. Box 7726 Austin, TX 78713-7726
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Office of Naval Research 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. / PROJECT NO. / TASK NO. / WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Electron Transfer Between Glucose Oxidase and Electrodes via Redox Mediators Bound with Flexible Chains to the Enzyme Surface

12. PERSONAL AUTHOR(S) Wolfgang Schuhmann, Timothy J. Ohara, Hanns-Ludwig Schmidt, Adam Heller

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Technical	FROM 7/1/89 TO 1/1/91	1991 1 11	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION
22a. NAME OF RESPONSIBLE INDIVIDUAL Adam Heller	22b TELEPHONE (Include Area Code) (512) 471-8874 22c. OFFICE SYMBOL

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION OF THIS PAGE

Unclassified

UNIT NO. 238 901207
Gal. 1 JA1C15M JA9015050 V113 1003

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Electron Transfer between Glucose Oxidase and Electrodes via Redox Mediators Bound with Flexible Chains to the Enzyme Surface

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Abstract: Electrical communication between redox centers of glucose oxidase and vitreous carbon electrodes is established through binding to oligosaccharides, at the periphery of the enzyme, ferrocene functions pendant on flexible chains. Communication is effective when the chains are long (>10 bonds), but not when the chains are short (<5 bonds). When attached to long flexible chains, the peripherally bound relays penetrate the enzyme to a sufficient depth to reduce the electron-transfer distances between a redox center of the enzyme and the relay and between the relay and the electrode, thereby increasing the rate of electron transfer.

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Introduction

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The redox centers of many enzymes are electrically insulated by thick protein or glycoprotein shells, preventing direct electrical communication between the centers and electrodes.[1] The rate of electron transfer between a redox center of an enzyme and an electrode is controlled by (a) the distance between the redox center and the electrode, (b) the potential difference between the redox center and the electrode, and (c) the reorganization energy associated with the electron transfer.[2] For enzymes such as glucose oxidase, with buried redox centers, diffusing redox mediators including O_2/H_2O_2[3] and ferrocene/ferricinium derivatives[4] have been used to shuttle electrons between enzyme redox center and electrodes. Leakage of ferrocene/ferricinium mediators from thin-film enzyme electrodes leads to their deterioration.[5] Leakage can be avoided through the use of soluble diffusing high molecular weight redox mediators, such as ferrocene-derivatized bovine serum albumin[6] and ferrocene bound to high molecular weight poly(ethylene glycol)[7] that can be confined within membranes having sufficiently small pores.

PAR06

Direct, i.e., not *diffusionally* mediated, electrical communication between a buried redox center of an enzyme and an electrode can be achieved through insoluble, electrode-attached redox polymers that penetrate the enzyme sufficiently deeply for electron exchange.[8] This route provides the significant advantage of eliminating the need for membrane containing the soluble macromolecular mediator. Yet another way to establish direct electrical communication between a buried redox center of an enzyme and an electrode is through covalently binding to the protein of the enzyme (well below its "periphery") electron relays.[9] For example, with glucose oxidase, a rather rigid glycoprotein with two identical polypeptide chains and a hydrodynamic radius of ~50 Å, the distances involved in electron transfer between the active sites and the electrode are shortened upon binding 12 or more ferrocenecarboxylic acid functions, through amide links, to the enzyme. Replacement of ferrocenecarboxylic acid by ferroceneacetic acid or ferrocenebutanoic acid enhances the kinetics of electron transfer.[9c,d] In the preparation of materials for affinity chromatography, redox-active species of enzymes, such as NAD^+/NADH, are bound to supports with long and flexible spacer chains. Such chains facilitate access of the active species to their specific binding sites.[10]

PAR09

We report here the modification of glucose oxidase by covalently binding of ferrocene derivatives, via spacer chains of different lengths, to sugar residues on its outer surface. We show that the length of the spacer chain has a crucial influence on the electrooxidation of the enzyme, i.e., on electron transfer from the reduced active site of the enzyme via the spacer chain attached ferrocenes to electrodes. This process is rapid only when the spacer chain is sufficiently long to allow the ferrocene to penetrate the enzyme sufficiently to approach the redox center.

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Experimental Section

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Chemicals. Glucose oxidase type X (EC 1.1.3.4, from *Aspergillus niger*, 128 units mg^{-1}), sodium *m*-periodate, sodium boron hydride, 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), 1,2-ethylenediamine, 1,3-diaminopropane, 1,6-diaminohexane, 1,8-diaminooctane, 1,10-diaminodecane, and diethylenetriamine were purchased from Sigma; ferrocene carboxaldehyde (98%) was obtained from Aldrich. (Aminoethyl)ferrocene was synthesized according to literature[11] and precipitated as the chloride salt. All other chemicals were of the best

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SEN15 9 available grade and used without further purification. Unless otherwise
4 noted, all experiments were performed at room temperature in a standard
15 aqueous buffer solution containing 100 mM phosphate and 200 mM
25 NaCl at pH 7.2.
PAR15
SEN03 1 **Electrodes and Equipment.** Electrochemical measurements were per-
SEN06 5 formed with an EG&G Princeton Applied Research 175 universal pro-
14 grammer, a Model 173 potentiostat, and a Model 179 digital coulometer.
SEN09 1 The signal was recorded on a Kipp and Zonen Y-Y-Y′ recorder. Glassy
SEN12 3 carbon rods (Sigradur, 3-mm diameter) sealed with epoxy resin into glass
14 were polished prior to use on a polishing cloth sequentially with alumina
26 of decreasing particle size (1, 0.3, 0.5 μm), sonicated, rinsed with distilled
SEN15 38 water, and then dried in air. A single-compartment electrochemical cell
6 was used with an aqueous KCl/saturated calomel (SCE) reference
SEN18 15 electrode and a platinum counter electrode. All potentials are referred
6 to this reference electrode (+244 mV vs NHE).
PAR18
SEN03 1 **Synthesis of Ferrocene Derivatives.** The ferrocene derivatives with
SEN09 6 different spacer lengths were synthesized as shown in Figure 1. A 4-fold FIG 1 (006,15-16)
4 excess of the appropriate diamine was heated in 100 mL of DMF to 100
18 °C, and 500 mg of ferrocenecarboxaldehyde dissolved in 50 mL of DMF
30 was added dropwise within 1 h to prevent formation of the bridged
SEN12 42 diferrocene compound. After another hour an excess of sodium boro-
9 hydride in water was dropped into the solution, and the reaction mixture
SEN15 21 was stirred for an additional hour at room temperature. The solvent
4 mixture was rotavaporated to dryness and the residue extracted with
14 dichloromethane and separated through a silica column (1.5 cm × 30
SEN18 25 cm). A first fraction—the bridged diferrocene—was eluted with di-
9 chloromethane, the main fraction with dichloromethane/methanol 10:1.
SEN21 1 The solvent was evaporated to dryness, the residue dissolved in diethyl
13 ether, and the hydrochloride precipitated by bubbling gaseous hydro-
SEN24 21 chloric acid through the solution. All compounds show the expected ^1H
8 NMR spectra.
PAR21
SEN03 1 **Preparation of Ferrocene-Modified Glucose Oxidase.** The oxidation
SEN06 4 of the enzyme-bound sugar residues was performed with sodium *m*-
SEN09 13 periodate according to established procedures.[12] The ferrocenes were FNT 12
5 attached to the aldehyde groups formed thus on the outer enzyme surface
17 via Schiff bases, which were reduced with sodium borohydride subse-
SEN12 26 quently (Figure 2). The modified enzyme was isolated from low mo- FIG 2 (009,28-29)
9 lecular weight compounds and desalted by gel chromatography (Sepha-
SEN15 17 dex G25 equilibrated with water; column 2.5 cm × 20 cm). The volume
4 was reduced by means of ultrafiltration through a membrane (Amicon
SEN18 14 PM30, MWCO 30000), and the modified enzyme was freeze-dried. To
3 verify that the unreacted ferrocenes were not electrostatically bound to
13 the enzyme, the freeze-dried product was redissolved and extracted with
23 copious amounts of a solution containing 0.1 M phosphate and 0.1 M
SEN21 35 NaCl at pH 7.1 in an ultrafiltration cell. After refreeze-drying, the
5 electrochemical characteristics of the modified enzyme were unchanged,
SEN24 13 confirming the absence of noncovalently bound ferrocenes. Determina-
2 tion of the amount of aldehyde groups at the enzyme surface was per-
SEN27 14 formed by a procedure of Sawicki et al.[13] The activity of the lyophilized FNT 13
7 enzymes was determined spectrophotometrically by the *o*-dianisidine/
SEN30 13 peroxidase assay.[14] The labeling of the enzyme with ferrocenes was FNT 14
10 evaluated by atomic absorption spectroscopy and by coulometry.
TXT09
SEN03 1 **Results and Discussion**
PAR24
SEN03 1 **Synthesis of Ferrocene-Labeled Glucose Oxidase.** Glucose
SEN06 3 oxidase (EC 1.1.3.4 from *Aspergillus niger*) is a dimer glycoprotein
SEN09 13 with a molecular mass of 186000 daltons. The oligosaccharide
4 chains, which form a hydrophilic periphery, represent ~12% of
SEN12 13 its weight. Oxidation of these with periodate[12] has been used to
11 provide peripheral aldehyde groups for the immobilization of
19 glycoenzymes to polymeric supports[15] or to electrode surfaces.[16] FNT 15, FNT 16
SEN15 1 Analogously, we have now applied this method to bind ferrocene
12 derivatives with different spacer lengths to the surface of glucose
SEN18 22 oxidase. The periodate oxidation of glucose oxidase was inves-
9 tigated with respect to the number of aldehyde functions obtained
SEN21 19 and the decrease of enzymatic activity during the reaction. As
3 expected, the aldehyde concentration increased when the reaction
SEN24 11 times were longer and the enzymatic activity decreased. Optimal
3 results were obtained at a reaction time of 1 h and a periodate
SEN27 16 concentration of >20 mM, the conditions of our experiments. The
3 number of aldehyde groups, introduced upon oxidation with 20
12 mM sodium periodate, was determined spectrophotometrically
18 after its reaction with 3-methyl-2-benzothiazolinone hydrazone
SEN30 24 hydrochloride, following a procedure of Sawicki et al.[13] Assuming
3 that the extinction coefficient reported for the hydrazones of
12 aldehydes formed from mannitol (ϵ = 95000 L mol^{-1} cm^{-1}) is
23 similar to that of the hydrazones of the oxidized enzyme, we
SEN33 34 estimate 6.4 aldehyde groups per enzyme molecule.[17] However, FNT 17
3 because polysaccharides do not react as completely as mono-
11 saccharides with this hydrazone, and because the extinction
19 coefficient for the aldehydes derived from mannitol is higher than
SEN36 29 that of other sugars, this estimate may be low. The functionalized

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enzyme used for the covalent binding of the different ferrocene compounds showed an activity of 66 units mg^{-1}.

PAR27

As the rate of electron transfer decays exponentially with the distance of the involved redox centers, a significant influence of the spacer length between enzyme surface and mediator on the electron-transfer properties of the modified enzyme in question was expected. To evaluate the effect of chain length on the effectiveness of electron transfer to electrodes, we prepared the series of ferrocene-derivatized enzymes shown in Table I (compounds 1–7). The amino-functionalized ferrocene derivatives have been synthesized through the reaction sequence shown in Figure 1 and purified by column chromatography. Following IO_4^- oxidation of the oligosaccharide residues on the enzyme, the resulting aldehyde groups were reacted with ferrocene amines, to form Schiff bases. These were reduced with $NaBH_4$ to the secondary amines (Figure 2). Binding of amino spacer modified ferrocene derivatives to the surface of the functionalized glucose oxidase did not lead to a further decrease of enzymatic activity (see Table I).

relay

TBL I (006,27-28)

PAR30

Electrochemical Investigations of Ferrocene-Modified Glucose Oxidase. The results of the electrochemical measurements are summarized in Figure 3 and Table I. The cyclic voltammograms shown in Figure 3 were run at 2 mg mL^{-1} concentration of the ferrocene-modified enzymes 1–7 in 0.1 M phosphate buffer (pH 7.2). The three-electrode cells were equipped with a glassy carbon (3-mm diameter) working electrode, a platinum wire counter electrode, and a KCl-saturated calomel reference electrode. Catalase was added to the solutions (200 units mL^{-1}) to decompose any hydrogen peroxide that might be formed in the presence of residual oxygen. Curve 1 of Figure 3 shows the cyclic voltammograms of a solution of compound 1 in buffer (a) without glucose and (b) with 40 mM glucose. Curves 2 and 3 show the cyclic voltammograms observed under identical conditions for compounds 2 and 4, respectively. The limiting currents, normalized for the amount of attached ferrocene, increase with chain length (Table I). Notable enhancement of the catalytic current is observed in compound 7, where i = 6.5 μA, i.e., the current density reaches 90 μA cm^{-2}.

FIG 3 (006,12-13)

PAR33

Electron-Transfer Model. A peripherally attached redox mediator may accept electrons through either an intramolecular or an intermolecular process (Figure 4), acting in the latter as a conventional diffusing mediator. For example, mediation by ferrocene-modified albumin has been reported.[6] The dominance of the intramolecular electron-transfer process in the case of enzymes with long chains was established through the following experiment. Enzymes 1 and 4 were partially deactivated by 6 M urea (4 h, 25°), and then separated from the urea by gel-permeation chromatography. Their catalytic currents i' (Table II) were measured at an enzyme concentration of 1 mg mL^{-1} under conditions identical with those for i_{cat} in Table I. Then 1 mg mL^{-1} native glucose oxidase was added, and the catalytic current (i''_{cat}, Table II) was determined. If the process were entirely intermolecular, i''_{cat} would have been equal to or greater than i_{cat}, because the concentration of the electron-transfer mediator is unchanged and both the concentration and relative catalytic activity of the enzyme are increased (note in Table I that 1 and 4 retain, respectively, 0.27 and 0.45 of the native enzyme's activity). If the process were entirely intramolecular, addition of native enzyme would not have changed the catalytic current seen with the deactivated enzyme (i'_{cat}, Table II). Measurement of the catalytic current in the presence of deactivated 1 and 4 with native enzyme added shows that in the case of 1, where the chain is short, the current approaches i_{cat} for the enzyme prior to deactivation, i.e., that the process of electron transfer either has a substantial intermolecular component or is entirely intermolecular. For compound 4, made with long chains, i''_{cat}, the current observed with the partially deactivated enzyme plus native enzyme (470 nA), remains much below the 2800-nA catalytic current of the enzyme prior to its partial deactivation and is only marginally higher than the 350-nA current of the partially deactivated enzyme (Table II). This indicates that when the spacer chain is long the process is dominantly intramolecular. We thus conclude that the increase in catalytic currents with increase in chain length (Table I and Figure 3) originates in enhanced intramolecular electron transfer from the enzyme's redox centers to the chain-attached mediator and, via the mediator, to the electrode. Our observations do not allow us to define the extent of electron transfer by a dynamic process, where the chain-pendant mediator swings "in"

FIG 4 (006,19-20)

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and "out" of the enzyme, and a static process, where the relay is reasonably stationary, i.e., is bound by hydrophobic or electrostatic interaction to a specific region in the protein.

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SEN00 **Acknowledgment.** We thank Dr. B. A. Gregg for the preparation of (aminoethyl)ferrocene and many helpful discussions. The work at the University of Texas at Austin is supported by the Office of Naval Research, the Welch Foundation, and the Texas SEN09 Advanced Research Program. The work at the Technical University of Munich is supported by the Bundesministerium für Forschung und Technologie (BMFT), Projektträger Biotechnologie, FRG. This collaborative study was performed at the SEN12 University of Texas.

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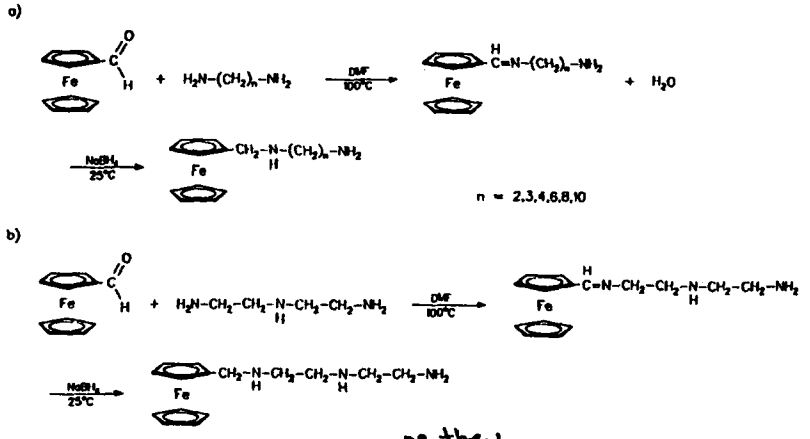
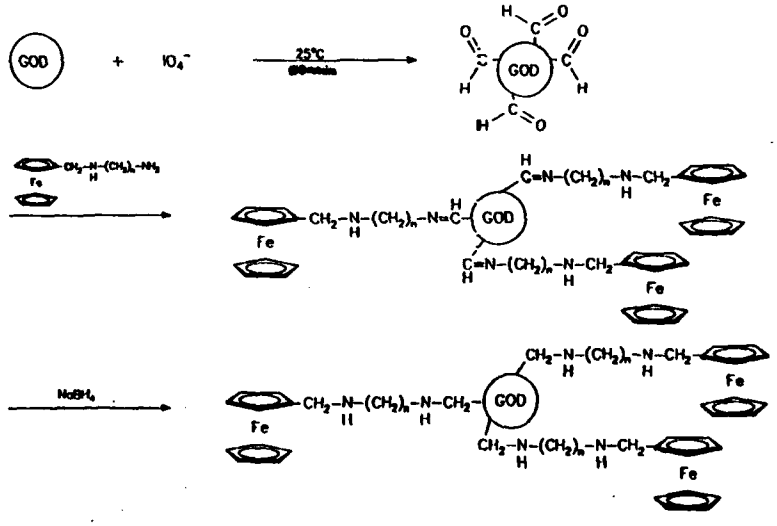


CAP00 1 **Figure 1.** Synthesis of ferrocene amines with spacer chains ~~for the~~ separating the redox and amine functions.



CAP00 1 **Figure 2.** Preparation of glucose oxidase modified by peripherally bound ferrocenes.

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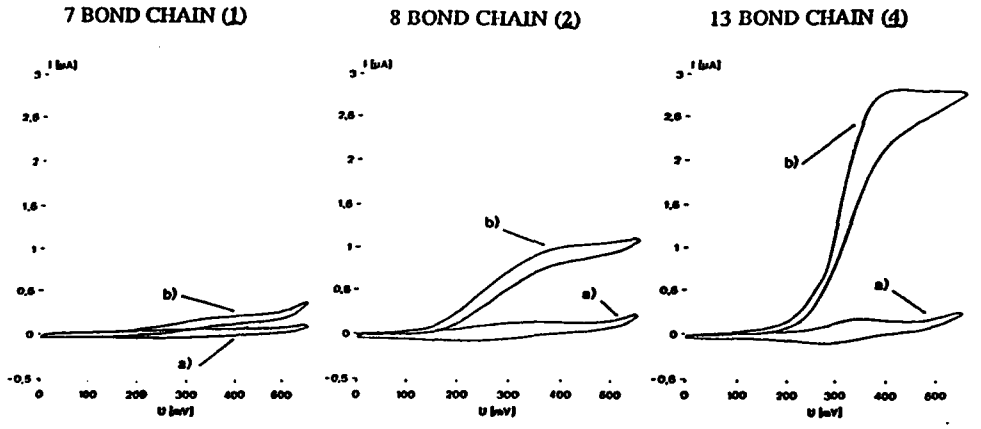

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Figure 3. Effect of the chain length connecting peripherally bound ferrocene to glucose oxidase on the electrocatalytic glucose oxidation current. Curves a represent oxidation currents in the absence of glucose; curves b represent currents at 40 mM glucose. All solutions contain 2 mg mL^{-1} of one of the modified enzymes, 0.1 M phosphate buffer (pH 7.2), and 200 units/mL^{-1} catalase; 3-mm-diameter glassy carbon disks; all potentials vs SCE; scan rate 10 mV s^{-1}.

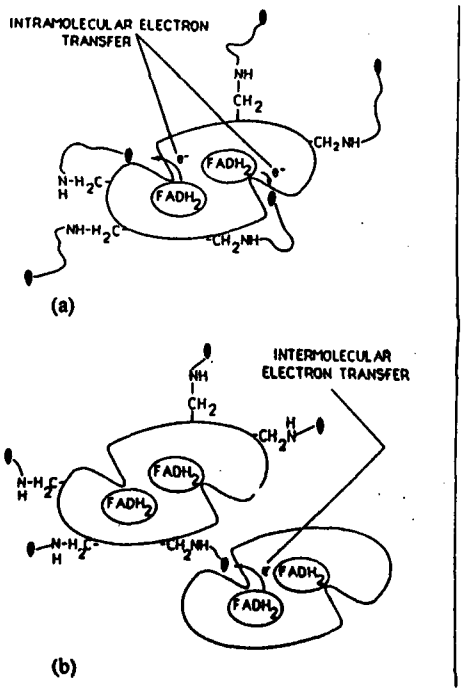

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Figure 4. (a) Intramolecular and (b) intermolecular electron transfer via chain-attached mediators.

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TTL20 **Table I. Effect of the Spacer Chain Length on the Catalytic Current of Ferrocene-Modified Glucose Oxidase**

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	no.	compound	bonds	i_{cat},[a] nA	$[Fc]_{rel}$[b]	$i_{cat}/[Fc]_{rel}$	rel enzyme activ $[O_2]$[c]
ROW50	1	Enz-CH_2-NH-$(CH_2)_3$-NH-CH_2-Fc	7	200	1.50 ± 0.20	400 ± 160	0.27
ROW60	2	Enz-CH_2-NH-$(CH_2)_3$-NH-CH_2-Fc	8	1010	1.00 ± 0.10	1010 ± 100	0.38
ROW70	3	Enz-CH_2-NH-$(CH_2)_6$-NH-CH_2-Fc	11	1190	1.00 ± 0.10	1190 ± 120	0.45
ROW80	4	Enz-CH_2-NH-$(CH_2)_6$-NH-CH_2-Fc	13	2800	1.00 ± 0.10	2800 ± 280	0.41
ROW90	5	Enz-CH_2-NH-$(CH_2)_{10}$-NH-CH_2-Fc	15	2680	1.00 ± 0.10	2680 ± 270	0.49
ROW100	6	Enz-CH_2-NH-$(CH_2)_2$-Fc	5	460	0.75 ± 0.25	600 ± 200	0.33
ROW110	7	Enz-CH_2-NH-$[(CH_2)_2$-$NH]_2$-CH_2-Fc	10	3200	1.00 ± 0.10	3200 ± 320	0.36

FNT120 [a] Catalytic glucose oxidation current on 3-mm-diameter glassy carbon electrodes at 0.35 V (SCE). [b] Coulometrically determined relative number of ferrocenes per enzyme. [c] Hydrogen peroxide rate of formation, measured relative to the native glucose oxidase rate.

AID00 INITIAL TABLE WIDTH IS DOUBLE COLUMN

TTL20 **Table II. Catalytic Current of Partially Deactivated Ferrocene-Modified Enzymes**

HDB40

	no.	compound	bonds	i_{cat},[a] nA	i'_{cat} (deactiv),[b] nA	i''_{cat} (deactiv + native enz),[c] nA
ROW50	1	Enz-CH_2-NH-$(CH_2)_3$-NH-CH_2-Fc	7	200	120	170
ROW60	4	Enz-CH_2-NH-$(CH_2)_6$-NH-CH_2-Fc	13	2800	350	470

FNT70 [a] Catalytic current for modified enzyme from Table I. [b] Catalytic current for modified, then partially deactivated enzyme. [c] Catalytic current of (b) after addition of an equal amount (1 mg mL^{-1}) of native glucose oxidase.

AID00 INITIAL TABLE WIDTH IS SINGLE COLUMN

The number of words in this manuscript is 2896.

The manuscript type is A.

Running Heads

Electron Transfer between Glucose Oxidase and Electrodes

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Text Page Size Estimate = 2.4 Pages

Graphic Page Size Estimate = 1.5 Pages

Total Page Size Estimate = 3.9 Pages